A

FISH & RICHARDSON P.C.

4225 Executive Square
Suite 1400
La Jolla, California
92037

Telephone
619 678-5070

Facsimile
619 678-5099

Web Site
www.fr.com

Frederick P. Fish
1855-1930

W.K. Richardson
1859-1951

December 16, 1998

Attorney Docket No.: 07349/019001

Box Patent Application
Assistant Commissioner for Patents
Washington, DC 20231

BOSTON
NEW YORK
SILICON VALLEY
SOUTHERN CALIFORNIA
TWIN CITIES
WASHINGTON, DC

Presented for filing is a new provisional-to-utility patent application of:

Applicant: ETHAN BIER, KWEON YU
Title: PEPTIDE INHIBITOR OF TGF-ß GROWTH FACTORS

Enclosed are the following papers, including all those required to receive a filing date under 37 CFR §1.53(b):

	Pages
Specification	21
Claims	3
Abstract	1
Declaration	[To Be Filed At A Later Date]
Drawing(s)	6

Enclosures:
- Postcard.

Under 35 USC §119(e)(1), this application claims the benefit of prior U.S. provisional application 60/069,701, filed 12/16/97.

This application is entitled to small entity status. A small entity statement will be filed at a later date.

"EXPRESS MAIL" Mailing Label Number EM320639039US
Date of Deposit December 16, 1998
I hereby certify under 37 CFR 1.10 that this correspondence is being deposited with the United States Postal Service as **"Express Mail Post Office To Addressee"** with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Duncan Clark

Duncan Clark

Basic filing fee	$ 760.00
Total claims in excess of 20 times $11.00	66.00
Independent claims in excess of 3 times $41.00	123.00
Multiple dependent claims	0.00
Total filing fee:	$ 949.00

A check for the filing fee is enclosed. Please apply any other required fees or any credits to deposit account 06-1050, referencing the attorney docket number shown above.

If this application is found to be INCOMPLETE, or if a telephone conference would otherwise be helpful, please call the undersigned at 619/678-5070.

Kindly acknowledge receipt of this application by returning the enclosed postcard.

Please send all correspondence to:

Stacy L. Taylor
Fish & Richardson P.C.
4225 Executive Square, Suite 1400
La Jolla, CA 92037

Respectfully submitted,

Stacy L. Taylor
Reg. No. 34,842

Enclosures
77706.LJ1

PROVISIONAL-TO-UTILITY APPLICATION FOR UNITED STATES PATENT IN THE NAME OF

Ethan Bier, Kweon Yu

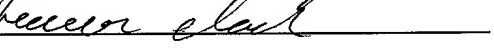
FOR

PEPTIDE INHIBITOR OF TGF-β GROWTH FACTORS

Stacy L. Taylor, Esq.
FISH & RICHARDSON
4225 Executive Square, Suite 1400
La Jolla, CA 92037
(619) 678-5070 voice
(619) 678-5099 fax

Date of Deposit: December 16, 1998
*I hereby certify under 37 C.F.R. 1.10 that this correspondence is being deposited with the United States Postal Service as "**Express Mail Post Office To Addressee**" with sufficient postage on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.*

Duncan Clark



Docket No. 07349/019001

Express Mail Label No.: EM320639039US

PEPTIDE INHIBITOR OF TGF-β GROWTH FACTORS

BACKGROUND OF THE INVENTION

This application claims priority to Provisional Application No. 60/069,701, filed 12/16/97.

1. *Field of the Invention*

The invention relates to a peptide and homologues thereof which inhibit the activity of TGF-β family growth factors in vertebrates, as well as TGF-β-like growth factors in *Drosophilia*. Specifically, the invention relates to a highly potent fragment of the product of the *Drosophilia short gastrulation* gene (*Sog*).

2. *History of the Related Art*

During vertebrate development, growth factors in the TGF-β superfamily control a number of events in tissue differentiation and morphogenesis. Included in the TGF-β superafamily are the bone morphogenic proteins, which promote the growth of new bone tissue and differentiation of osteoblasts. Examples of members of this family of BMPs are BMP-4 and BMP-7, which suppress neurogenesis during early embroynic development and are active in aspects of adult physiology.

BMP-4 has been highly conserved through evolution and has a functional and structural homologue in *Drosophilia*, known as Dpp. BMP-4 can substitute for Dpp in *Drosophilia* (Padgett, *et al., Proc.Natl.Acad.Sci. USA,* 90:2905-2909 (1993)) and Dpp is active in vertebrate tissues (Sampath, *et al., Proc.Natl.Acad.Sci.USA*, 90:6004-6008 (1993)). In vertebrates, chordin is a high affinity BMP-4 binding protein which inhibits BMP-4 and BMP-7 activity (Sasai, *et al., Cell*, 779-790 (1994)); in flies, the short gastrulation (Sog) protein inhibits Dpp activity (Francois, *et al., Genes & Dev.*, 8:2602-2616 (1994)). Again, chordin and Sog are functional and structural homologues

(Schmidt, *et al., Development*, 121:4319-4328 (1995) and Francois, *et al., Cell*, 80:19-20 (1995)). In particular, Sog inhibits BMP-4 activity in vertebrates in a manner similar to chordin (Schmidt, *et al., id.)*. It is highly probable that Sog inhibits the activity of other members of the TGF-β family such as BMP-7, which is also inhibited by chordin.

Abnormal activity on the part of BMP-4 has been linked to human cancer, including osteosarcoma and certain leukemias. Interestingly, over-expression of BMP-4 (which is potentiated by BMP-7) has also been shown to stimulate the onset of alopecia (male pattern baldness) in mice, perhaps due to an effect on hair follicle development. Control of such activity can have therapeutic benefit in these and other conditions related to abnormalities in the functioning of TGF-β family growth factors, especially on the part of BMP-4.

SUMMARY OF THE INVENTION

The invention provides a highly potent inhibitor of TGF-β growth factor activity, with particular impact on BMP-4. In particular, the invention identifies a fragment of the *Drosophilia* Sog protein which has an unexpectedly high level of Dpp inhibitory activity as compared to the intact, wild-type protein.

Based on the known homologies between Dpp and BMP-4, as well as between Sog and chordin, together with other supportive data discussed below, it is predictable that the Sog fragment of the invention (hereafter, "Super-Sog") functions as an inhibitor of BMP-4 and BMP-7 activity in vertebrates. Surprisingly, Super-Sog has a broader scope of activity than wild-type Sog in the sense that mutant phenotypes not produced by Sog inhibition of Dpp are produced in response to Super-Sog. This phenomenon suggests that Super-Sog is more potent than Sog and/or that it affects additional receptor-ligand interactions not affected by the wild-type protein, such as those mediated by activin, a vertebrate endocrine regulator whose *Drosophilia* homologue mediates wing development.

The invention therefore provides Super-Sog (SEQ.ID.No. 1; amino acids 1-292 of Sog) and active variants thereof. Such variants include SEQ.ID.No. 3, a recombinant Super-Sog peptide which includes 33 amino acids encoded by the pUAS expression vector; SEQ.ID.No. 6, a Super-Sog peptide which includes a mutation (W→A) in the CR-1 sequence; and SEQ.ID.No. 7, a Super-Sog peptide which terminates 5' of the CR-1 sequence. Such variants also include Super-Sog with 5' modifications, such as modifications to the Tolloid protease cleavage site, addition of other peptides and inclusion of additional 5' regions of Sog (e.g., CR-2).

The invention further provides pharmaceutical compositions of Super-Sog and methods for their use. Especially useful among the pharmaceutical compositions are those which are prepared so as to increase the bioavailability of Super-Sog *in vivo* by, for example, protecting the peptide against unintended proteolysis.

Methods for use of Super-Sog include its therapeutic use in arresting the development of male pattern baldness, assisting in the treatment of cancer (e.g., osteosarcomas) and inhibiting TGF-β growth factor (e.g., BMP-4, BMP-7 and activin) mediated suppression of neurogenesis to, for example, enhance the viability of fetal nervous tissue grafts in the treatment of neurodegenerative disorders such as Alzheimer's Disease, Parkinson's Disease and Huntington's Disease.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a diagram of the nucleotide sequence (SEQ.ID.No. 1) coding for a Super-Sog polypeptide, whose amino acid sequence (SEQ.ID.No. 2) is shown beneath the nucleotide codons in the Figure. Transmembrane (TM) and CR-1 regions of the coding sequence and peptide are indicated in the right margin of the FIGURE.

FIGURE 2 is a compilation of the nucleotide sequence (SEQ.ID.No. 1) coding for a Super-Sog polypeptide and 33 amino acids encoded by the pUAS expression vector (SEQ.ID.No. 3), coded by the nucleotide sequence following the NotI restriction site.

FIGURE 3 is the nucleotide sequence (SEQ.ID.No. 6) coding for a Super-Sog peptide which includes a mutation (W→A) in the CR-1 sequence.

FIGURE 4 is the nucleotide sequence (SEQ.ID.No. 7) coding for a Super-Sog peptide which is modified 5' of the NotI restriction site sequence of SEQ.ID.No. 3.

FIGURE 5 is a line comparison demonstrating partial sequence homology between Super-Sog (SEQ.ID.No. 4) and another Dpp antagonist in *Drosophilia*, noggin (SEQ.ID.No. 5).

FIGURE 6 is the full-length nucleotide sequence (SEQ.ID.No. 8) coding for wild-type Sog.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

I.

Structure and Activity of Super-Sog

The *Sog* coding sequence (Francois, *et al., Genes & Dev.*, 8:2602-2616 (1994)) contains 4 cysteine-rich repeat sequences (CR-1 through CR-4). The CR repeats are defined by a fixed spacing of 10 cysteine residues (excepting CR-4, which lacks the ninth cysteine) and share other many amino acids in common. CR-1 is located immediately after the putative transmembrane domain of the Sog protein (Fig. 1), while CR-2 through CR-4 are located closer to the coding region for the carboxyl terminus of the protein (Fig. 6).

Given their similarity in structure, it would be reasonably expected that any *Dpp* inhibitory activity conferred on the Sog protein by the CR repeats would be comparable in quality. It was therefore a surprise to find that a peptide encoded by CR-1 (Super-Sog; SEQ.ID.No.1) has greater Dpp inhibitory activity in certain respects than wild-type Sog.

In particular, both Sog and Super-Sog inhibit Dpp-mediated activation of the *rhomboid (rho)* gene (which stimulates wing vein development in pupal *Drosophilia*). However, as between Super-Sog and Sog, only the former inhibits Dpp-mediated expression of the *spalt* gene which encodes a transcription factor that influences wing vein placement in an earlier stage of *Drosophilia* development (Example I). Thus, Super-Sog appears to be at least as potent, and broader in spectrum, a TGF-β family growth factor inhibitor than wild-type Sog.

II.

Pharmaceutical Uses for Super-Sog

Given the apparent proficiency of Sog to inhibit the activity of TGF-β family growth factors (e.g., BMP-4 and BMP-7; collectively, "Target Factors") in vertebrates, Super-Sog should be useful in suppressing, for example, BMP-4 signalling in conditions where hyperactivity in such signalling is

causative. The relatively small size of the Super-Sog peptide, coupled with its potency as compared to Sog, makes Super-Sog a particularly attractive compound for pharmaceutical use.

Clinically, Super-Sog will be useful in the same therapies which are or may be practiced with inhibitors of TGF-β activity, especially activity mediated by BMP-4 signalling. Thus, therapeutic uses for Super-Sog include, but are not limited to, stimulation of bone growth and repair through osteogenesis, inhibiting BMP-4 suppression of neurogenesis at the site of nervous tissue grafts (e.g., fetal tissue grafts in Huntington's patients), stimulating neurogenesis as a adjunct to therapy of neurodegenerative disorders (e.g., in Huntington's, Parkinson's and Alzheimer's disease) and suppression of BMP-4 signalling of cancerous growth.

II.

Methods for Manufacture of Super-Sog

Super-Sog is prepared as a purified peptide fragment from Sog (e.g., SEQ.ID.No. 2), expressed as a recombinant peptide using, for example, the coding sequences set forth in SEQ.ID.Nos. 1, 3, 6 or 7, or synthesized chemically. Techniques for production of peptides according to each of these methods are well-known in the art and so will only be described briefly here.

In this respect, it will be appreciated by those of ordinary skill in the art that additions, substitutions and deletions of amino acids in the Super-Sog peptide sequences specifically described herein may be made without adversely altering the activity of the peptide. For example, a U→A substitution between the first two cysteines in CR-1 does not affect the activity of Super-Sog. Similarly, extending the length of Super-Sog 3' through the transmembrane and intracellular regions of Sog does not affect the activity of the peptide (although the increased length may decrease its bioavailability). Thus, in general, deletions and mutations made 3' to the CR-1 region in Super-Sog do not appear to adversely affect its activity.

Similarly, adding the CR-2 region or other constructs, such as a polyA sequence, to SuperSog 5' of the CR-1 region does not appear to adversely affect its activity. However, deletion of the entire

peptide 5' of the CR-1 region negates the activity of Super-Sog. Thus, it appears that at least a portion of the SuperSog peptide 5' to the CR-1 region (between CR-1 and CR-2) is necessary to the Target Factor inhibitory activity of the peptide.

The term "purified peptide" refers to a compound which is substantially free of other compounds with which it may normally be associated *in vivo*. In the context of the invention, the term refers to homogenous Super-Sog, which homogenicity is determined by reference to purity standards known to those of ordinary skill in the art (e.g., purity sufficient to allow the N-terminal amino acid sequence of the protein to be obtained). Super-Sog may be obtained from embryonic insects, such as *Drosophilia,* or insect cell lines, and purified to homogenicity using means known to those skilled in the art, such as affinity chromatography.

Recombinant Super-Sog can also be produced *in vitro* or *in vivo* through expression of a polynucleotide sequence which encodes Super-Sog (e.g., SEQ.ID.No. 1). In general, prokaryotes are used for cloning of DNA sequences in constructing recombinant expression vectors. For example, *E. coli* K12 strain 294 (ATCC Accession No. 31446) may be particularly useful. Prokaryotes also are used for expression. The aforementioned strain, as well as *E. coli* W3110 (ATTC Accession No. 27325), bacilli such as *Bacillus subtilus*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcescans*, and various *pseudomonas* species may also be used for expression.

Non-viral plasmid vectors which may be used in the invention contain promoters and control sequences which are derived from species compatible with the host cell. The vector ordinarily carries a replication site as well as marker sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, *et al., Gene*, 2:95, 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain or be modified to contain promoters and other control elements commonly used in recombinant DNA construction.

Expression may also be achieved using RNA or DNA viruses, including retroviruses, adenoviruses, herpes virus, vaccinia and adeno-associated viruses. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), human immunodeficiency virus (HIV-1) and Rous Sarcoma Virus (RSV). Adeno-associated viruses are especially useful for their stable expression and relative lack of adverse side effects when delivered *in vivo*.

A number of viral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting one or more sequences of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target specific.

Further, in addition to prokaryotes, eukaryotic microbes such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used eukaryotic microorganism for use in *in vitro* expression of polynucleotides, although a number of other strains are commonly available.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman, *et al., J. Biol. Chem.*, 255:2073, 1980) or other glycolytic enzymes (Hess, *et al. J. Adv. Enzyme Reg.* 7:149, 1968; and Holland, *Biochemistry*, 17:4900, 1978) such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degraded enzymes associated with nitrogen metabolism,

metallothionine, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Yeast enhancers also are advantageously used with yeast promoters.

Super-Sog can also be readily synthesized by conventional techniques, such as the solid phase synthesis techniques as described in Gutierrez, *et al., FEBS Letters*, 372:39-43 (1995), the disclosure of which is incorporated herein by this reference to illustrate knowledge in the art concerning techniques for the production of synthetic peptides.

Briefly, commonly used methods such as t-Boc or Fmoc protection of alpha-amino groups are suitable for use in synthesizing Super-Sog of the invention. Both methods involve stepwise syntheses whereby a single amino acid is added at each step starting from the C terminus of the peptide (see, Coligan, *et al., Current Protocols in Immunology,* Wiley Interscience, 1991, Unit 9). Peptides of the invention can also be synthesized by various well known solid phase peptide synthesis methods, such as those described by Merrifield *(J. Am. Chem. Soc.*, 85:2149, 1962), and Stewart and Young (*Solid Phase Peptides Synthesis*, Freeman, San Francisco, 1969, pp 27-62), using a copoly (styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer. On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 1/4-1 hours at 0°C. After evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution which is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on a "SEPHADEX G-15" or "SEPHAROSE" affinity column. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which can then be characterized by such standard techniques as amino acid analysis, thin layer chromatography, high performance liquid chromatography (HPLC), ultraviolet absorption spectroscopy, molar rotation, solubility, and quantitated by the solid phase Edman degradation.

The synthesis objective is to produce peptides whose charge distribution is similar to that in the native sequence. The selection of resins and post-synthesis treatments for each peptide will therefore be optimized for this result. In particular, resins that yield a free carboxy group are useful to generate peptides representing the C-terminal of a protein. Subsequently, the N-terminal will be acetylated.

Resins that yield an aminated C-terminal are useful to generate internal peptides and peptides representing the N-terminal region. For generation of internal sequence peptides, the N-terminal is acetylated, whereas for generation of N-terminus peptides, the N-terminal is free.

In order to increase the bioavailability of Super-Sog, the peptide may be synthesized using standard Fmoc or t-Boc chemistries with amino acid derivatives in D-conformation. Alternatively, sequences with reduced peptide bonds in positions susceptible to proteolysis may be synthesized according to, for example, Meyer et al., *J.Med.Chem.,* 38:3462-3468 (1995) (incorporated herein for reference). Briefly, such peptides are synthesized using a Fmoc/tert-butyl strategy, and the $Y(CH_2NH)$ bonds, or reduced bonds, are introduced via reductive alkylation of the N-terminal amino group of the growing peptide with a Fmoc-Na-protected amino aldehyde.

To increase the efficacy of selected peptides so they can exert their physiological effect for longer periods of time, the following refinements to Super-Sog may be made using techniques which those of ordinary skill in the art will be familiar with or can readily ascertain.

The acetylation of an N-terminal amino group or the choice of N-terminal amino acid can dramatically improve the α-helical stability (Chakrabartty, *et al., Proc. Natl. Acad. Sci. USA* 90:11332-11336 (1993); Jarvis, *et al., J. Biol. Chem.* 270:1323-1331 (1995)) and biological activity of a peptide (Dooley, *et al., Science* 266:2019-2022 (1994)). N-terminal acetylation has also been described as a factor which contributes to the stabilization of coil forming peptides (Greenfield, *et al., Protein Science* 3:402-410 (1994)) and to increase resistance to proteolytic degradation by exopeptidases (Abiko, *et al., Chem. Pharm. Bull.* 39:752-756 (1991)). Super-Sog may therefore be modified to have enhanced activity and stability by acetylation of their N-termini.

D-isomers of Super-Sog are also desirable for their resistance to proteolytic degradation *in vivo*. It is well recognized that L-bond peptides are susceptible to proteolytic degradation, restricting their application as drugs. However, this obstacle has been successfully bypassed in some cases by synthesizing analogues which contain D-bond amino acids or non-natural amino acids. The addition of a single D-amino acid at the C-terminal position is enough to enhance the resistance to proteolytic

degradation by exopeptidases, without significantly altering the secondary structure of the peptide (Abiko, *supra*). Resistance to endopeptidases can be achieved by including individual non-cleavable non-peptidic bonds in points in the peptide sequence that are specially sensitive to enzymatic degradation (Meyer, *et al., J. Med. Chem.* 38:3462-3468 (1995); Guichard, *et al., Peptide Research* 7:308-321 (1994)). Reverse amide bonds Y[NHCO], reduced amide bonds Y[CH_2NH] or retro-reduced bonds Y[$NHCH_2$] can be used as surrogates of the amide link [CONH] in ESUPs of the invention. Reduced amide links are preferred, since they result only in minor destabilization of a-helices (Dauber-Osguthorpe, *et al., Int. J. Pep. Prot. Res.* 38:357-377 (1991)). In Sog, the cleavage site for the Tolloid protease near the COOH end of CR-1 is a convenient site for modification of Super-Sog to increase its resistance to proteolytic degradation.

Alternatively, Super-Sog can be synthesized in an all-D-conformation. All-D-peptides can be equally active as the original all-L-peptides (Merrifield, *et al., Ciba Foundation Symposium* 186:5-20 (1994); Wade, *et al., Proc. Natl. Acad. Sci. USA* 87:4761-4765 (1990)), capable of successfully resisting enzymatic degradation and less immunogenic than their all-L-analogues (King, *et al., J. Immunol.* 153:1124-1131 (1994)).

III.

Pharmaceutical Compositions of Super-Sog and Super-Sog Kits

Pharmaceutically useful compositions of Super-Sog are prepared by mixing the peptide with physiologically acceptable carriers. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the particular protein with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients.

Such compositions may be lyophilized for storage and will be reconstituted according to pharmaceutically acceptable means; i.e., suitably prepared and approved for use in the desired application. A sodium chloride free buffer is preferred for use as a reconstituting agent. Whatever

its form, the composition product will be placed into sterile containers (e.g., ampules) for storage and transportation.

For targeted delivery of Super-Sog, a colloidal dispersion system is useful. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al., Trends Biochem. Sci.*, 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes encoding the antisense polynucleotides at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al., Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingo-lipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is

saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

Super-Sog kits are also provided by the invention. In diagnostic and research applications such kits may include any or all of the following: assay reagents, buffers and Super-Sog protein and/or homologues, Super-Sog coding recombinant expression vectors, oligonucleotide and other hybridization probes and/or primers, and/or a suitable assay device. A therapeutic product may include sterile saline or another pharmaceutically acceptable emulsion and suspension base for use in reconstituting lyophilized Super-Sog or Super-Sog antibody suspensions, suitably labeled and approved containers of Super-Sog or Super-Sog antibody compositions, and kits containing these products for use in connection with the diagnostic kit components as described above.

Such a kit may also comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes, and the like, each of the container means comprising one of the separate elements to be used in the method.

For example, one of the container means may comprise a hybridization probe that is or can be detectably labelled. A second container may comprise a cell lysis buffer. The kit may also have containers holding nucleotide(s) for amplification of the target nucleic acid sequence and/or a container comprising a reporter-means, such as a biotin-binding protein, such as avidin or streptavidin, bound to a reporter molecule, such as an enzymatic, fluorescent, or radionuclide label.

IV.

Methods and Dosages for In vivo Delivery of Super-Sog

A "therapeutically effective dosage" of Super-Sog composition in a pharmaceutically acceptable carrier will be that dosage which produces a therapeutic benefit in a host that can also be provided by another BMP-4 inhibitor (e.g., Sog), but is provided in lieu of such other inhibitor. In particular, because Super-Sog's affinity for Dpp is in the picomolar range, it is predictable to those of ordinary skill in the art that dosing regimes (including dosing concentrations and schedules) applied to other pharmaceutical compounds which target receptor-ligand interactions with affinities in the picomolar range will be useful for SuperSog (depending on the patient's condition and the medical judgment of the clinician).

Analogous examples include neurotransmitter antagonists, serotonin uptake inhibitors and GABA antagonists (see, e.g., Boritzki, *et al., Invest.New Drugs,* 14:295-303 (1996) and Coy, *et al., J.Biol.Chem.,* 25:14691-14697 (1989)). Based on these analogies, and evidence obtained through administration of SuperSog to flies and frogs, suitable concentrations of SuperSog for use in therapeutic applications range from about 0.01 to 200 nanomolar, with suitable concentrations for many applications falling within the range of about 0.1 to 50 nanomolar.

Depending on the desired clinical result, the dosage will be administered by means best suited to reach target tissue; e.g., for the scalp, a topical route of administration may be preferred while for neurogenesis, grafting of cells transformed with a recombinant expression vector encoding Super-Sog may be a better choice (see, e.g., commonly assigned U.S. Patent Nos 5,082,670; 5,529,774 and

5,650,148, incorporated herein for reference regarding skill in the art concerning *ex vivo* gene therapy and cell grafting to nervous tissue in vertebrates).

Super-Sog can also be labeled with a paramagnetic isotope for purposes of *in vivo* imaging, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR) techniques. Usually gamma and positron emitting radioisotopes are used for camera imaging and paramagnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

Depending on the desired clinical target, Super-Sog may be delivered to a host using any available method and route suitable for drug delivery, including *ex vivo* methods (e.g., delivery of cells incubated or transfected with a Super-Sog encoding polynucleotide) as well as systemic or localized routes. However, those of ordinary skill in the art will appreciate that parenteral methods of administration, especially by non-systemic routes, will generally be preferred for avoidance of peptide degradation *in vivo*.

Intranasal administration means include inhalation of aerosol suspensions or insufflation of the polynucleotide compositions of the invention. Nebulizer devices suitable for delivery of polynucleotide compositions to the nasal mucosa, trachea and bronchioli are well-known in the art and will therefore not be described in detail here. For general review in regard to intranasal drug delivery, those of ordinary skill in the art may wish to consult Chien, *Novel Drug Delivery Systems*, Ch. 5 (Marcel Dekker, 1992).

Examples of means for delivering drugs to the skin are topical application of a suitable pharmaceutical preparation, transdermal transmission, injection and epidermal administration.

For transdermal transmission, absorption promoters or iontophoresis are suitable methods. For review regarding such methods, those of ordinary skill in the art may wish to consult Chien, *supra* at Ch. 7. Iontophoretic transmission may be accomplished using commercially available "patches" which deliver their product continuously via electric pulses through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical

compositions in relatively great concentrations, permits infusion of combination drugs and allows for contemporaneous use of an absorption promoter.

An exemplary patch product is the LECTRO PATCH trademarked product of General Medical Company of Los Angeles, CA. This product electronically maintains reservoir electrodes at neutral pH and can be adapted to provide dosages of differing concentrations, to dose continuously and/or to dose periodically. Preparation and use of the patch should be performed according to the manufacturer's printed instructions which accompany the LECTRO PATCH product; those instructions are incorporated herein by this reference.

Intraparenchymal administration to the brain, as well as administration to the central nervous system, may be performed by known means including grafting drug expressing transformed host cells onto the target tisue and injection of the drug by microsyringe directly into tissue. Those of skill in the art may wish to consult *Neural Grafting in the Mammalian CNS*, Bjorklund and Steveni, eds., (1985) as well as U.S. Patent Nos. 5,082,670 and 5,529,774, each of which is incorporated herein to illustrate the level of skill in the art of drug delivery to the brain and CNS.

Systemic administration involves invasive or systemically absorbed topical administration of pharmaceutical preparations. Topical applications as well as intravenous and intramuscular injections are examples of common means for systemic administration of drugs.

V.

Diagnostic Uses for Super-Sog

Hyperactive BMP-4 signalling is indicative of diminished Super-Sog-like antagonism of BMP-4. Measurement of Super-Sog-like activity *in vivo* therefore would be of diagnostic value with respect to conditions causatively associated with hyperactive BMP-4 signalling. For *in vitro* measurements, anti-Super-Sog antibodies may be used diagnostically (e.g., to detect Super-Sog in a biological cell sample or monitor the level of its expression). A suitable cell sample may be derived from tissue

biopsies, sputum specimens, blood specimens or urinary specimens. Germline cells may be obtained from any convenient source, such as skin, blood, or hair follicles.

Super-Sog and its structural homologue in vertebrate species may be detected and/or bound using Super-Sog antibodies in either liquid or solid phase immunoassay formats (when bound to a carrier). Examples of well-known carriers for use in solid-phase assay formats include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding antibodies, or will be able to ascertain such, using routine experimentation. Examples of types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format.

Specific examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Using the Super-Sog antibodies of the invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Super-Sog antibodies and peptides can be used to measure Target Factor inhibition and binding using Super-Sog antibodies (see, e.g., the binding assay protocols described in Grazioli, *et al., Int.J.Immunopharma.*, 14:637-642 (1992) and Hosang, *J.Cell.Biochem.*, 29:265-273 (1985)). Those of skill in the art will know, or can readily discern other immunoassay formats without undue experimentation.

Super-Sog and Super-Sog homologues may also be detected in cell samples through hybridization of labelled probes or primers which bind the gene which encodes Super-Sog. Hybridization probes and primers generally do not encode Super-Sog but nonetheless are capable of hybridizing with DNA encoding Super-Sog. Such probes and primers are usually oligonucleotides; i.e., either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

Such oligonucleotides may be detectably labelled with a detectable substance such as a fluorescent group, a radioactive atom or a chemiluminescent group by known methods and used in conventional hybridization assays.

The Super-Sog antibodies of the invention may also be detectably labelled. There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, fluorescent compounds, colloidal metals, chemiluminescent compounds, and bio-luminescent compounds. Those of ordinary skill in the art will know of other suitable labels for binding to the anti-Super-Sog antibodies of the invention, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the Super-Sog antibodies of the invention can be done using standard techniques common to those of ordinary skill in the art. Another labeling technique which may result in greater sensitivity consists of coupling the antibodies to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use haptens for this purpose such as biotin, which reacts with avidin.

Monitoring of the effect of Super-Sog applied therapeutically may be made by measuring BMP-4 levels at the onset, and through the duration, of therapy. Such tests may be performed on fluid or cell samples *in vitro*, or through *in vivo* imaging. In using the anti-Super-Sog antibodies of the invention for the *in vivo* detection of antigen having a peptide of the invention, the detectably labeled monoclonal antibody is given in a dose which is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled Super-Sog antibody is administered in sufficient quantity to enable detection of the site having cells which express Super-Sog.

For this purpose, the concentration of detectably labeled Super-Sog antibody which is administered should be sufficient such that it is detectable compared to the background. Further, it is desirable that the detectably labeled Super-Sog antibody be rapidly cleared from the target tissue in order to give the best target-to-background signal ratio.

For *in vivo* imaging, the type of detection instrument available is a major factor in selecting a given detectable label; e.g., a radioisotope. The radioisotope chosen must have a type of decay which is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized.

Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the 140-250 keV range, which may be readily detected by conventional gamma cameras. Typical examples of metallic ions which can be bound to the ESUPs of the invention are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, ^{90}Y, and ^{201}Tl.

As a rule, the dosage of detectably labeled Super-Sog antibody for *in vivo* diagnosis will vary depending on such factors as age, sex, and extent of disease of the individual. The dosage of antibody can vary from about 0.01 mg/m^2, to about 500 mg/m^2, preferably 0.1 mg/m^2 to about 200 mg/m^2, most preferably about 0.1 mg/m^2 to about 10 mg/m^2. Such dosages may vary, for example, depending on whether multiple injections are given, tissue, and other factors known to those of skill in the art.

Examples illustrating the construction and use of the invention are provided below. These examples do not limit the scope of the invention, which is defined by the appended claims. Standard abbreviations (e.g., "ml" for milliliters) are used in the examples unless otherwise noted.

EXAMPLE I

Effect of Super-Sog on Wing Structure in Drosophilia

As a convenient reference point for the effect of Super-Sog on Dpp activity in *Drosophilia* (modeling the capacity of Super-Sog to modulate BMP-4 activity in vertebrates, if not the outcome of that modulation), Dpp signalling consequences in wing morphogenesis are scored for the development of one or more of four phenotypes characteristic of Dpp suppression: (1) missing

sections of wing veins; (2) thickened wing veins; (3) decreased distances separating wing veins; and (4) decreased wing length, each as compared to wing phenotypes in normal flies (see, e.g., Yu, *et al., Development,* 122: 4033-4044 (1996), especially FIG. 1 comparing Dpp-active and Dpp-suppressed wing phenotypes in adult flies).

Wild-type Sog antagonizes Dpp to block wing formation (phenotype 1). For comparison, a broader spectrum vertebrate BMP-4 antagonist (noggin) which shares a fairly high degree of coding sequence homology with Super-Sog (FIG. 3) blocks some wing formation (phenotype 1), shortens the distance between wing veins through fusion (phenotype 3) and produces shorter wings (phenotype 4) in *Drosophilia*.

Super-Sog elicits all four Dpp-suppression phenotypes. Its effect on the development of phenotypes 1, 3 and 4 is similar to the effects produced by noggin, although Super-Sog can stimulate fusion of all 5 *Drosophilia* wing veins while noggin influences fusion of only 2 sets of the veins (vein 2 with 3 and vein 4 with 5). Preliminary results indicate comparable results (in different phenotypic models) are produced as a consequence of Super-Sog suppression of BMP-4 in frogs.

The invention having been fully described, modifications thereto may be apparent to those of ordinary skill in the art. All such modifications are included within the scope of the claimed invention.

SUMMARY OF SEQUENCES

SEQ.ID.No. 1 is the nucleotide sequence which codes for a Super-Sog polypeptide.

SEQ.ID.No. 2 is the predicted amino acid sequence of Super-Sog based on SEQ.ID.No. 1.

SEQ.ID.No. 3 is a compilation of the nucleotide sequence (SEQ.ID.No. 1) coding for a Super-Sog polypeptide and 33 amino acids of the pUAS expression vector (SEQ.ID.No. 3), coded by the nucleotide sequence following NotI.

SEQ.ID.No. 4 is a partial nucleotide sequence for *Super-Sog* encompassing a region of homology to *noggin*.

SEQ.ID.No. 5 is a partial nucleotide sequence for *noggin* encompassing a region of homology to *Super-Sog*.

SEQ.ID.No. 6 is the nucleotide sequence coding for a Super-Sog peptide which includes a mutation (W→A) in the CR-1 sequence.

SEQ.ID.No. 7 is the nucleotide sequence coding for a Super-Sog peptide which is modified 5' of the NotI restriction site sequence of SEQ.ID.No. 3.

SEQ.ID.No. 8 is the full-length nucleotide sequence coding for wild-type Sog.

What is claimed is:

CLAIMS

1. A polynucleotide comprising the nucleotides of SEQ.ID.No. 1.

2. A recombinant expression vector containing the polynucleotide of Claim 1.

3. A peptide comprising the amino acids of SEQ.ID.No. 2.

4. A pharmaceutical composition comprising the peptide of Claim 3.

5. Monoclonal antibodies having binding specificity for the peptide of Claim 3.

6. Polyclonal antibodies having binding specificity for the peptide of Claim 3.

7. A polynucleotide comprising the nucleotides of SEQ.ID.No. 3.

8. A recombinant expression vector containing the polynucleotide of Claim 7.

9. A peptide encoded by the polynucleotide of Claim 7.

10. A pharmaceutical composition comprising the peptide of Claim 9.

11. A polynucleotide comprising the nucleotides of SEQ.ID.No. 6.

12. A recombinant expression vector containing the polynucleotide of Claim 11.

13. A pharmaceutical composition comprising the peptide of Claim 12.

14. Monoclonal antibodies having binding specificity for the peptide of Claim 12.

15. Polyclonal antibodies having binding specificity for the peptide of Claim 12.

16. A polynucleotide comprising the nucleotides of SEQ.ID.No. 7.

17. A recombinant expression vector containing the polynucleotide of Claim 16.

18. A peptide encoded by the polynucleotide of Claim 16.

19. A pharmaceutical composition comprising the peptide of Claim 18.

20. Monoclonal antibodies having binding specificity for the peptide of Claim 18.

21. Polyclonal antibodies having binding specificity for the peptide of Claim 18.

22. A method for suppressing abnormally hyperactive BMP-4 signalling in a vertebrate host comprising administering a therapeutically effective dosage of the peptide of Claim 3 to the host.

23. A method for suppressing abnormally hyperactive BMP-4 signalling in a vertebrate host comprising administering a therapeutically effective dosage of the peptide of Claim 9 to the host.

24. A method for suppressing abnormally hyperactive BMP-4 signalling in a vertebrate host comprising administering a therapeutically effective dosage of the peptide of Claim 12 to the host.

25. A method for suppressing abnormally hyperactive BMP-4 signalling in a vertebrate host comprising administering a therapeutically effective dosage of the peptide of Claim 18 to the host.

26. A method for detecting Super-Sog-like activity in a host comprising measuring the level of Super-Sog or a Super-Sog homologue in a host tissue.

ABSTRACT OF THE DISCLOSURE

A potent peptide inhibitor (SuperSog) of TGF-β family growth factor signalling, peptide variants thereof and nucleotide coding sequences therefor are provided by the invention. The Super-Sog peptide comprises a fragment of the *Drosophilia short gastrulation (Sog)* gene which includes the CR-1 cysteine-rich repeat of *Sog*. Methods and compositions for use of Super-Sog in therapeutic and diagnostic applications are also provided.

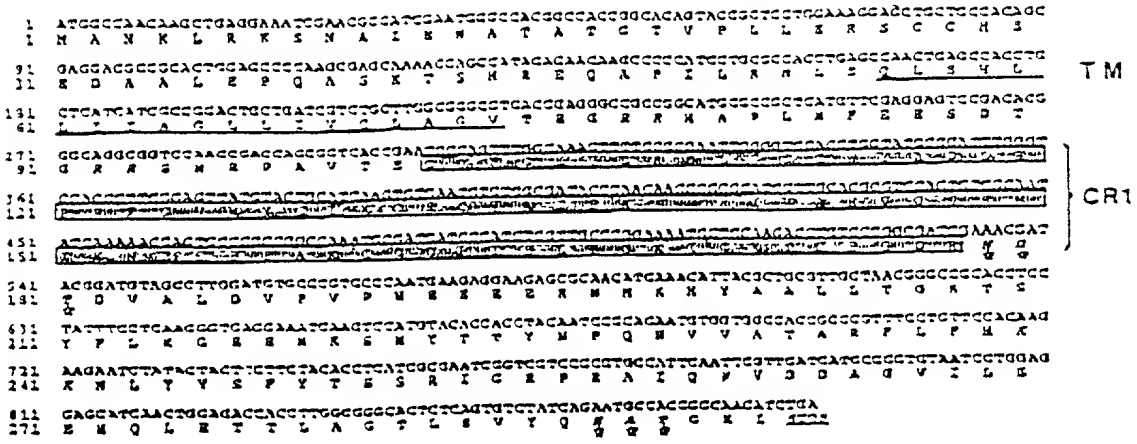


FIGURE 1

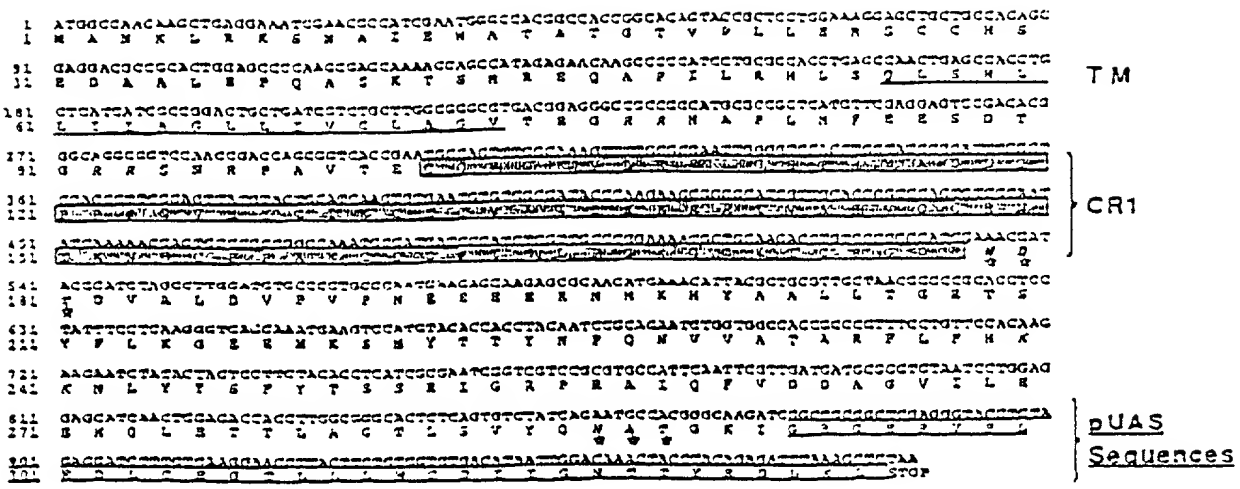


FIGURE 2

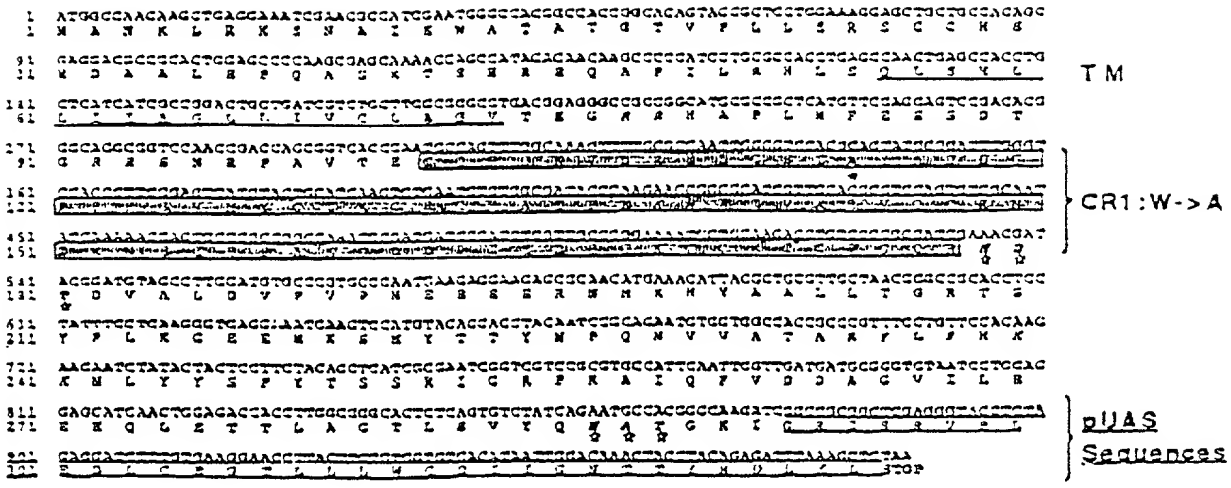


FIGURE 3

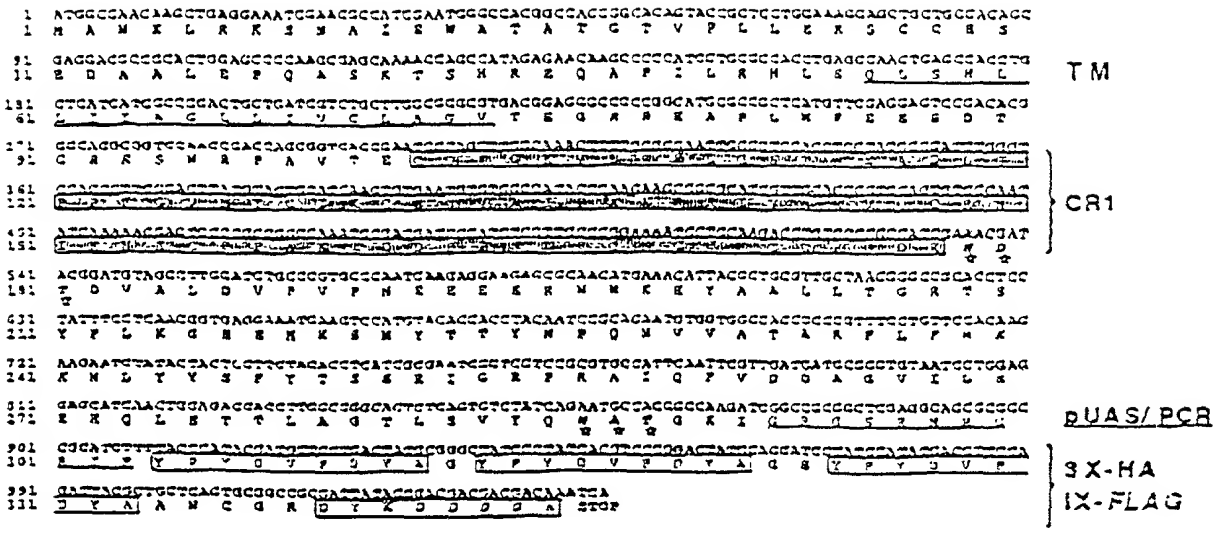


FIGURE 4

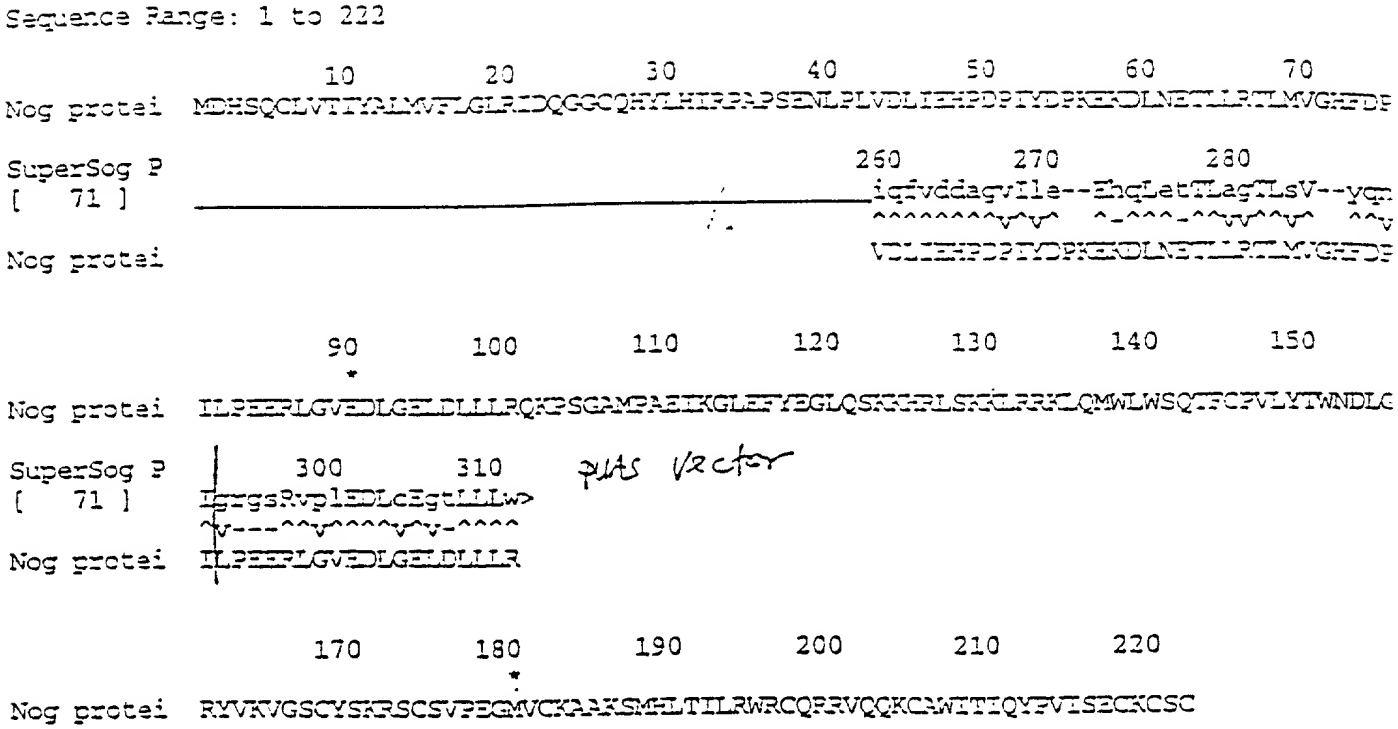

Sequence Range: 1 to 222
10 20 30 40 50 60 70
Nog protei MDHSQCLVTTYRLMVFLGLRIDQGGCQHYLHIRPAPSENLPLVDLIEHPDPTYDPKEKDLNETLLRTLMVGHFDP
SuperSog P
[71]
260 270 280
iqfvddagvIle--EhqLatTLagTLsV--yqn
Nog protei VDLIEHPDPTYDPKEKDLNETLLRTLMVGHFDP
90 100 110 120 130 140 150
Nog protei ILPEERLGVEDLGELDLLLRQKPSGAMPAEIKGLEFYEGLQSKKHRLSKGLRRFLQMWLWSQTFCPVLYTWNDLG
SuperSog P
[71]
300 310
IgrgsRvplEDLcEgtLLLw>
Nog protei ILPEERLGVEDLGELDLLLR
pIAS Vector
170 180 190 200 210 220
Nog protei RYVKVGSCYSKRSCSVPEGMVCKAAKSMHLTILRWRCQRRVQQKCAWITIQYFVISECKCSC

FIGURE 5

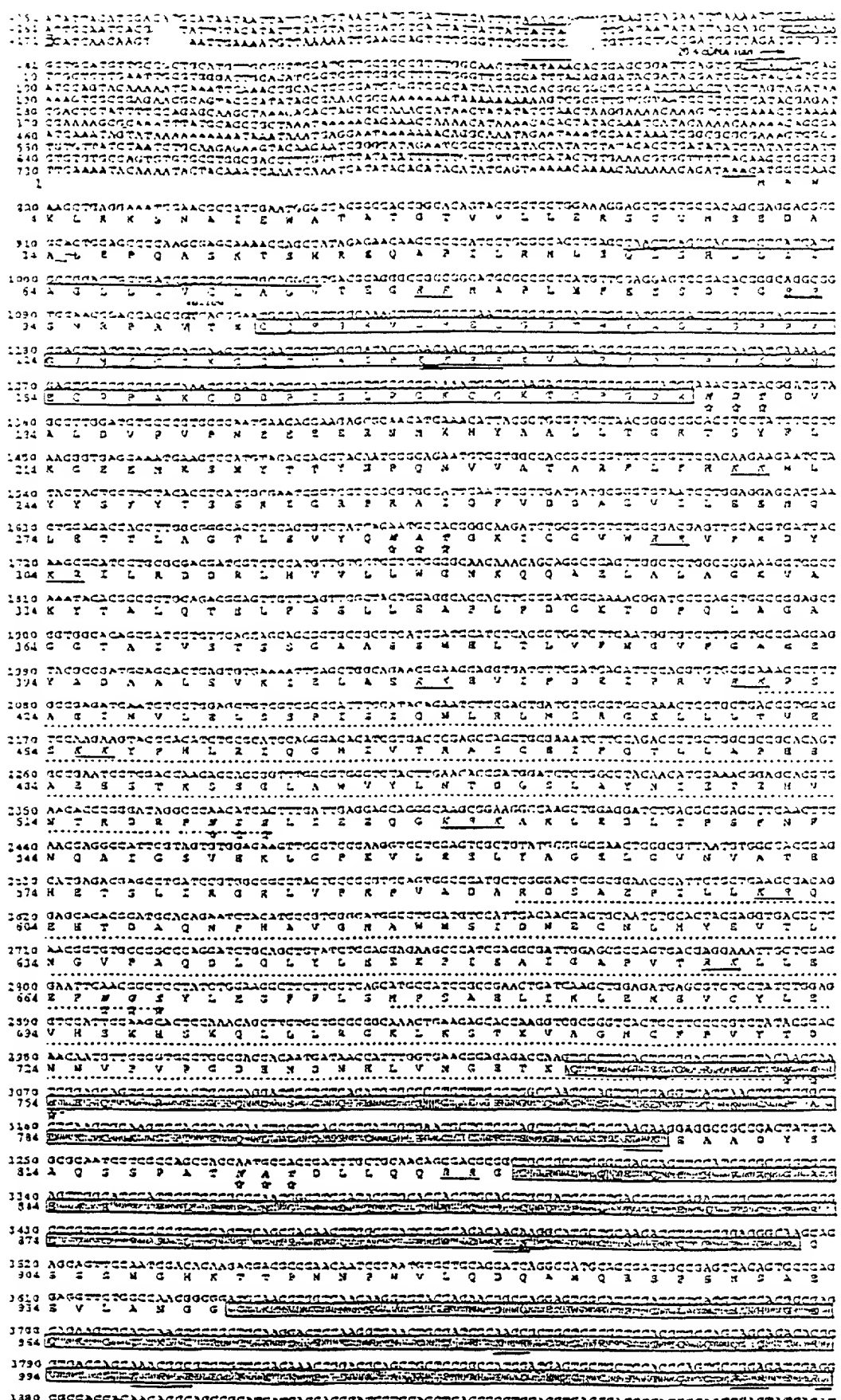


FIGURE 6

PRINT OF DRAWINGS
AS ORIGINALLY FILED

United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division

Application deficiencies were found during scanning:

☐ Page(s)__________ of NO DECLARATION were not present for scanning.
(Document title)

☐ Page(s)__________ of ________________________ were not present for scanning.
(Document title)

☐ Scanned copy is best available.